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Purification and Crystallizatin of L-Ornithine: α-Ketoglutarate δ-Aminotransferase from *Corynebacterium sepedonicum*

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INTRODUCTION

L-Ornithine δ-aminotransferase (L-ornithine:2-oxo-acid aminotransferase (EC 2.6.1.13)) catalyzes the δ-transamination of L-ornithine with α-ketoglutarate to produce L-glutamate and glutamate-γ-semialdehyde, which is spontaneously converted to Δ^1-pyrroline-5-carboxylate. The enzyme widely occurs in animals[1,2], higher plants[3,4] and microorganisms[5,6]. This enzyme has been purified to homogeneity from rat liver and kidney, and the rat liver enzyme has been studied enzymologically in detail,[7-13] but only little attention has been given to the microbial enzyme.

Recently, we purified L-ornithine: α-ketoglutarate δ-aminotransferase of *Bacillus sphaericus* to homogeneity and crytallized it.[14] We here describe the purification and crystallization of the enzyme from *Corynebacterium sepedonicum* and some of its properties.

MATERIALS AND METHODS

Pyridoxal 5′-phosphate was obtained from Kyowa Hakko Kogyo, Tokyo; DEAE-cellulose from Serva, Heidelberg; Sephacryl S-200 from Pharmacia, Uppsala; and polyethylenimine from Nakarai Chemicals, Kyoto. Hydroxyapatite was prepared by the method of Tiselius *et al.*[15]

Disc gel electrophoresis was performed by the procedure of Davis.[16]

The enzyme was assayed by determining L-glutamate (Method A) or Δ^1-pyrroline-5-carboxylate (Method B) produced. *Method A.* L-Glutamate in the supernatant solution was determined with ninhydrin after separation by circular chromatography.[17] *Method B.* To 0.5 ml of the supernatant solution were added 0.1 ml of 0.8 M KOH, 1.0 ml of 0.2 M potassium phosphate buffer (pH 8.0) and 1.0 ml of 25 mM *o*-aminobenzaldehyde in 10 % ethanol.[18] The mixture was incubated at 37°C for 30 min to develope a yellow color, and the absorbance was measured at 440 nm with a Hitachi UV-VIS 139 spectrophotometer. The standard

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reaction system consisted of 20 μmol of L-ornithine, 20 μmol of potassium α-keto-glutarate, 0.1 μmol of pyridoxal 5′-phosphate, 100 μmol of potassium phosphate buffer (pH 8.0) and enzyme in a final volume of 1.0 ml. Enzyme was replaced by water in a blank. After the mixture was incubated at 37°C for 20 min, the reaction was terminated by addition of 0.1 ml of 25 % trichloroacetic acid followed by centrifugation. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol of L-glutamate per min. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.*[19] with serum albumin as a standard.

RESULTS AND DISCUSSION

Purification of the enzyme. *Corynebacterium sepedonicum* IFO 3306 was grown in a medium composed of 0.1 % L-ornithine·HCl, 0.5% peptone, 0.2% glycerol, 0.2 % K_2HPO_4, 0.2 % KH_2PO_4, 0.2 % NaCl and 0.05 % yeast extract (pH 7.2). The culture was grown at 30°C 18 h under aeration. The harvested cells were washed twice with 0.85 % NaCl solution. All subsequent operations were at 0–5°C. The buffers used contained 10^{-5}M pyridoxal 5′-phosphate and 0.01 % 2-mercaptoethanol.

Step 1. Enzyme extraction. The washed cells (about 2 Kg, wet weight) were suspended in 20 mM potassium phosphate buffer (pH 7.4) containing 0.01 % 2-mercaptoethanol, and subjected to sonication for 20 min in a 19 KHz oscillator followed by centrifugation. The supernatant solution was used as a cell-free extract.

Step 2. Polyethylenimine treatment. To the cell-free extract was added 0.1 ml of 10 % polyethylenimine solution (pH 7.4) per 100 mg of the protein with stirring. After 30 min, the precipitate was removed by centrifugation. The supernatant solution was brought to 80 % saturation with ammonium sulfate. The precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.4) and then dialyzed for 30 h against four changes of 20 volumes of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

Step. 3. First DEAE-cellulose column chromatography. The enzyme solution was placed on a DEAE-cellulose column (10.1 × 70 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer and then with the buffer containing 0.15 M NaCl, the enzyme was eluted with the buffer supplemented with 0.20 M NaCl. The active fractions were combined and brought to 80 % saturation with ammonium sulfate. The precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.4), and the enzyme solution was dialyzed against 100 volumes of the same buffer.

Step 4. Second DEAE-cellulose column chromatography. The enzyme solution was subjected to DEAE-cellulose column (6.1 × 59 cm) in the same manner as mentioned above. The active fractions were concentrated by ammonium sulfate (80 % saturation) and dissolved in 1 mM potassium phosphate buffer (pH 7.4) followed by dialysis against 100 volumes of the same buffer.

Step 5. Hydroxyapatite column chromatography. The enzyme solution was applied to a

hydroxyapatite column (4.2×22 cm) equilibrated with 1 mM potassium phosphate buffer (pH 7.4). After the column was washed with 20 mM potassium phosphate buffer (pH 7.4), the enzyme was eluted with 40 mM potassium phosphate buffer (pH 7.4). The active fractions were collected and concentrated by ammonium sulfate (80 % saturation). The precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 8.5) containing 0.1 M NaCl and dialyzed against the same buffer.

Step 6. DEAE-Sephadex A-50 column chromatography. The enzyme solution was chromatographed on a DEAE-Sephadex A-50 column (1.2×8.4 cm) equilibrated with the dialysis buffer. After application of the enzyme and washing of the column with 10 mM Tris-HCl buffer (pH 8.5) containing 0.10 M NaCl, the enzyme was eluted with the buffer supplemented with 0.12 M NaCl. The active fractions were concentrated by ultrafiltration and then by addition of ammonium sulfate (80 % saturation). The precipitate was dissolved in a small volume of 20 mM potassium phosphate buffer (pH 7.4).

Step 7. Sephacryl S-200 column chromatography. The enzyme was applied to a Sephacryl S-200 column (1.5×120 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4) and eluted with the same buffer. The active fractions were concentrated by addition of ammonium sulfate (80 % saturation) and dissolved in a small volume of 20 mM potassium phosphate buffer (pH 7.4). Approximately 170-fold purification was achieved with an over-all yield about 10 %. A protocol of the purification is presented in a Table I.

Table I. Purification of L-ornithine: α-ketoglutarate δ-aminotransferase

Step	Total protein (mg)	Total units	Specific activity	Yield (%)
1. Crude extract	130,000	39,000	0.30	100
2. Polyethylenimine	79,300	33,300	0.42	85.4
3. First DEAE-cellulose	17,000	24,400	1.44	62.6
4. Second DEAE-cellulose	1,950	15,600	8.00	40.0
5. Hydroxyapatite	250	11,700	46.8	30.0
6. DEAE-Sephadex A-50	120	6,100	50.8	15.6
7. Sephacryl S-200	74.7	3,900	52.2	10.0

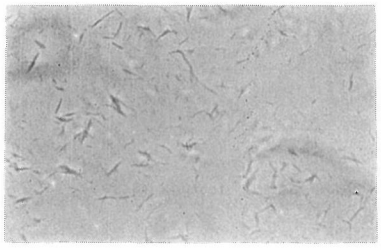

Fig. 1. Crystals of L-ornithine: α-ketoglutarate δ-aminotransferase of *Corynebacterium sepedonicum.*

Cystallization. Ammonium sulfate was added gradually to the enzyme solution until the solution became slightly turbid. The pH of the solution was kept constant at 7.2-7.4 with 14 % NH_4OH solution. On standing for a few days crystals formed. The crystals took the form of small needles (Fig. 1).

Properties of the enzyme. The purified enzyme was shown to be homogeneous by the criteria of disc gel electrophoresis (Fig 2). The molecular weight of the enzyme was determined to be approximately 81,000 by the gel filtration method of Andrews.[20] The value is closely similar to the that of the *Bacillus sphaericus* enzyme,[14] but is smaller than a half of that of the rat liver enzyme (mol. weight; 180,000)[12]. The enzyme shows absorption maxima at 278, 340 and 420 nm with an absorption ratio of 100:20:5 (Fig. 3). No appreciable spectral shifts occurred on varying the pH (5.0–9.0).

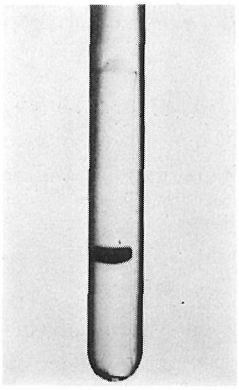

Fig. 2.

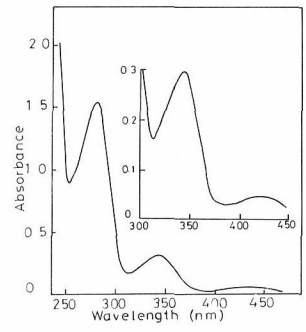

Fig. 3

Fig. 2. Disc gel electrophoresis of L-ornithine: α-ketoglutarate δ-aminotransferase. The crystalline enzyme preparation (50 μg) was electrophoresed under the conditions of Davis.[16]

Fig. 3. Absorption spectrum of L-ornithine: α-ketoglutarate δ-aminotransferase. Absorption spectrum of the enzyme was measured in 0.02 M potassium phosphate buffer (pH 7.4).

The aminotransferase catalyzes the transfer of δ-amino group of L-ornithine to α-ketoglutarate. None of D-ornithine, L-lysine, D-lysine, L-arginine, L-citrulline, glycine, L-alanine, L-aspartate, L-α-aminobutyrate, γ-aminobutyrate, β-alanine, taurine, putrescine and butylamine were the substrates. When L-ornithine was used as an amino donor, pyruvate, glyoxylate and oxaloacetate serve as very poor amino acceptors. α-Ketobutyrate, α-ketovalerate and β-phenylpyruvate were not the substrates. As reported previously,[14] the enzyme of *Bacillus sphaericus* also catalyzes the transamination between L-ornithine and α-ketoglutarate.

The enzyme has the maximum reactivity at about pH 8.0 and 45°C for the L-ornithine: α-ketoglutarate δ-transamination. The thermal stability of the enzyme was examined in the presence and absence of pyridoxal 5′-phosphate (Fig. 4). The enzyme was stable up to 65°C and inactivated beyond 70°C. The activity was increased to some extent by incubation with pyridoxal 5′-phosphate protected the

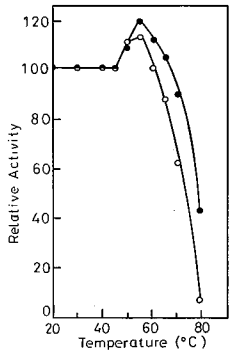

Fig. 4. Effect of temperature on the enzyme stability. The enzyme preparation was heated at the indicated temperature for 5 min (pH 8.0). The enzyme activity was assayed method B in the presence (—●—) or absence (—○—) of pyridoxal 5′-phosphate.

enzyme from thermal inactivation at a temperature above 80°C. The K_m values were calculated to be 1.8 mM for L-ornithine and 2.8 mM for α-ketoglutarate according to the method of Velick and Vavra.[21] These values are closely similar to those of *Bacillus sphaericus* enzyme[14] and mammalian enzyme.[12]

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